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A study on the effective substance of the Wu-tou formula based on the metabonomic method using UPLC-Q-TOF-HDMS

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The Wu-tou formula (WTF) is a Chinese medicine formula which has been applied to treat rheumatic arthritis (RA) and pain of joints for more than a thousand years. In this study, a pharmacodynamics combined urinary metabonomic study using UPLC-Q-TOF-HDMS was performed to assess the holistic efficacy of the Traditional Chinese Medicine (TCM) Wu-tou formula for treating RA in rats. Eighty male Sprague-Dawley rats were randomly divided into eight groups, named as the healthy control group (HG), the model group (AIA), the WTF group and five single herb groups. The treatment groups and the model group were induced for treating rheumatoid arthritis by using complete Freund's adjuvant. Histological results assessed the joint damage and several biochemical parameters such as IL-1β, TNF-α, SOD and MDA were used to evaluate inflammation injury and oxidative stress. Based on the results, a metabonomic investigation was conducted to study the mechanism of the WTF and single herb treatment groups for treating RA. Multivariate statistical analyses such as PCA and OPLS-DA were used to identify potential biomarkers in urine. As a result, twenty-six potential biomarkers have been found by comparison with the model and the WTF treatment group. The potential biomarkers mainly affect the phenylalanine, tyrosine and tryptophan biosynthesis pathway and the taurine and hypotaurine metabolism pathway. Aconiti Radix Preparata and Ephedrae Herba showed better effects on treating RA from the integrated evaluation by histological results, biochemical parameters and pattern recognition analysis. A comprehensive evaluation of the different therapeutic effects and the mechanism of each herb in the WTF for treating RA was performed in this research.

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Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease, involving the chronic and abnormal inflammatory disease of synovial joints, progressive destruction of cartilage and bone. The articular and extra-articular characteristics of RA include synovial hypertrophy, bone oedema, cartilage destruction, pulmonary fibrosis, pericardial inflammation, mononeuritis, an increase in aminotransferase concentrations and vasculitis.[1] Patients affected with RA usually have a risk factor to cardiovascular disease,[2] pulmonary embolism and deep vein thrombosis.[3] The cardiovascular risk is due to the high inflammatory which might be caused by RA.[4] The tumour necrosis factor α (TNF-α) and interleukin (IL) are the main cytokines in the process of synovitis and joint destruction.[5] Moreover, oxidative stress is an important factor for the pathogenesis of RA, which correlates with the degrees of inflammation and joint tissue damage.[6,7] Nevertheless, there is still no specific medicine to cure RA completely, thus it is necessary to further investigate the pathogenesis. In recent years, non-steroidal anti-inflammatory drugs (NSAIDs) and disease-modifying antirheumatic drugs (DMARDs) are commonly used. However, due to the side effects like liver and gastrointestinal disorders, the clinical uses of these drugs are limited. Consequently, a new therapeutic approach and new drugs are required for the treatment of RA. At present, traditional Chinese medicine (TCM) is widely used for the treatment of RA, because the formula of TCM usually contains multiple herbs and components, which might suit multiple targets of diseases. Therefore, the studies on TCM to treat RA are performed more and more extensively.

Chinese medicine theory considers that a formula containing several herbs could enhance the curative effect and reduce the toxicity due to their synergistic actions. For example, the Wu-tou

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Formula (WTF) is a classical formula mainly used to treat RA with excellent clinical effects, which contains five single herbs including Aconiti Radix Preparata (*Aconitum carmichaeli* Debx.), Ephedrae Herba (*Ephedra sinica* Stapf), Paeoniae Radix Alba (*Paeonia lactiflora* Pall), Astragali Radix (*Astragalus membranaceus* (Fisch.) Bge.) and Glycyrrhiza Radix Preparata (*Glycyrrhiza uralensis* Fisch.). Among which, Aconiti Radix Preparata as the main herb in the WTF plays a major role as an analgesic and to disperse moisture in joints, but its toxic effects were frequently reported since it has a narrow therapeutic range;[23] Ephedrae Herba assists Aconiti Radix Preparata to enhance the anti-rheumatic efficacy, because it can prompt the useful components to reach the joints; Astragali Radix strengthens the immune system in the body; Paeoniae Radix Alba reduces the pain of bones and muscles; Glycyrrhiza Radix Preparata produces anti-inflammatory effects, mainly reduces the toxicity of Aconiti Radix Preparata and so on.[8–10]

Metabonomics defined in 1999 by Nicholson is mainly used to study small-molecule metabolite profiles extracted from biofluids, cells or tissues. In recent years, it has been widely applied to toxicological survey, functional genomics, plants and microbes, clinical diagnostics and nutritional biochemistry.[11,12] TCM as a multi-compound and multi-target system is suitable for the method of metabonomics, owing to the commonness of the holistic value and integrity of dynamic alteration information of the body. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are two main approaches for the study of metabonomics. Compared with NMR, MS is more sensitive and versatile to the selective ionization method and the detector. MS conjugated with separation techniques such as gas chromatography-mass spectrometry (GC-MS), high-performance liquid chromatography-mass spectrometry (HPLC-MS), or capillary electrophoresis-mass spectrometry (CE-MS), could greatly increase the specificity and resolution, and reduce the complexity of the mass spectrum to be used for further processing.[13] Recently, high definition mass spectrometry (HDMS) coupled with ultra-performance liquid chromatography (UPLC) dominated the metabonomics arena, since this method could accurately identify the metabolite mass in a short time. In our earlier studies, the effect of the Wu-tou Formula on endogenous metabolites in urine of rats with RA was observed by the metabonomic method using ultra-performance liquid chromatography coupled with quadrupole time-of-flight high definition mass spectrometry (UPLC-Q-TOF-HDMS). The changes in the metabolite pathway after treatment with the WTF have been investigated using pattern recognition approaches.[8] However, the effective substance and the mechanism of the WTF have not been elucidated, and single herb actions on rats with RA as well as the metabolic pathways are not clear. In this study, the effects of five kinds of single herbs were evaluated by pathological variation of right hind joints and serum biochemical parameters on the model of adjuvant-induced arthritis (AIA) rats. On the basis of these studies, the metabolic pathways influenced by each herb were investigated using the metabonomic method to elucidate the integral mechanism for treating RA.

Results and discussion

Pharmacodynamics to assess the holistic efficacy of the WTF

Serum biochemical parameters. IL-1β and TNF-α played a central role in synovitis and joint destruction, and a quantitative analysis showed that they both are present in high concentration in synovial fluid and synovial tissue.[14] By measuring the concentrations of the two cytokines in serum *via* ELISA kits, it was found that the two cytokines up-regulated in the model group showed a significant inflammation response in rats with RA ($p < 0.01$) (Fig. 1). Compared with the model group, the level of the inflammatory cytokines in serum declined significantly ($p < 0.01$) in the WTF treatment group. The five single herb treatment groups also presented a certain degree of therapy efficiency. ZCW and MH significantly reduced the concentrations of TNF-α and IL-1β; HQ down-regulated the level of TNF-α and GC exhibited the effect of the reduced IL-1β level in serum. Of all the five herbs, ZCW and MH showed a better effect to reduce the two cytokines in serum.

Oxidative stress related to the pathogenesis of autoimmune disease like RA.

Peroxide radicals were generated in the inflammatory process and caused damage to the body. SOD induced the production of peroxide that was converted into hydrogen peroxide and oxygen, thereby clearing the radicals, and showed an anti-inflammatory effect. MDA is one of the most important peroxide products of membrane lipid, which could exacerbate damage to the membrane. Obviously, the AIA rats suffered oxidative stress with a low SOD activity and a high MDA level in serum (Table 1). By comparing the SOD and MDA concentrations between the treatment groups, ZCW, MH and BS showed the major antioxidant effect in the WTF as they could significantly decrease MDA concentration and increase SOD activity. HQ and GC showed less antioxidant capacity in the formula.

Histological results. As shown in Fig. 2A, there is no sign of inflammation in the right joint section of the healthy group,

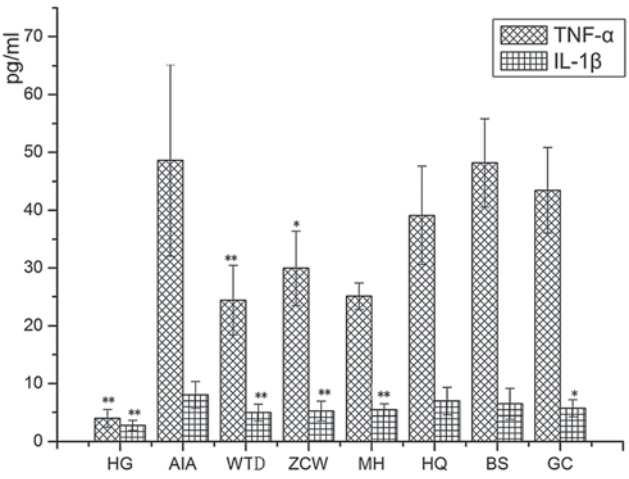

Fig. 1 Concentrations of IL-1β and TNF-α in serum of the study groups, after 3 week treatment.

Table 1 SOD activities and MDA contents in serum of different groups (mean ± SD)

	HG	AIA	WTF	ZCW	MH	BS	HQ	GC
SOD (U mL^{-1})	138.43 ± 3.56**	126.01 ± 5.22	137.40 ± 8.00**	132.98 ± 3.00**	133.43 ± 4.14**	133.10 ± 2.53**	129.16 ± 4.24	131.28 ± 5.54
MDA (nmol mL^{-1})	6.30 ± 1.02**	10.00 ± 1.80	7.67 ± 1.42**	7.63 ± 0.84**	7.99 ± 0.71*	7.55 ± 2.34**	8.61 ± 1.91	7.91 ± 0.88*

vs. model group, $^{**}p < 0.01$, $^{*}p < 0.05$.

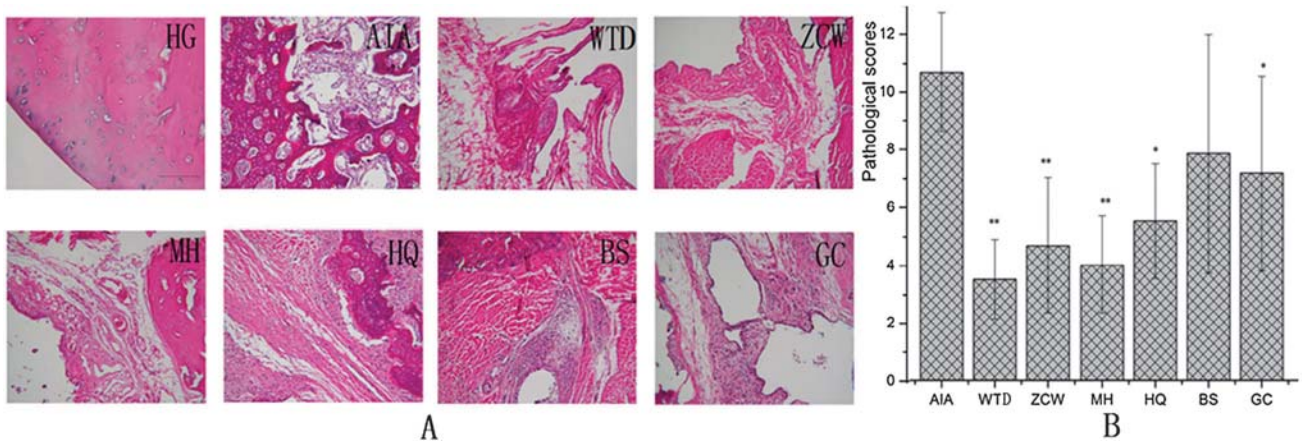

Fig. 2 (A) Histopathologic analyses of right hind joints with haematoxylin and eosin stain (original magnification 100 diameters); (B) 16 scores to evaluate the inflammatory levels, for inflammatory infiltration, synovial proliferation, cartilage erosion, and bone destruction from 0 to 3 for each aspect. ($^{*}p < 0.05$, $^{**}p < 0.01$, compared to the model group).

while the model group had serious synovial proliferation, cell inflammation, cartilage destruction and bone erosion. The synovial plica had mild hyperplasia of intra-articular in WTF, ZCW and MH groups, meanwhile synovial proliferation, cell infiltration and moderate inflammation appeared in BS, HQ and GC groups. A total of 16 scores were used to evaluate the inflammatory level, which included inflammatory infiltration, synovial proliferation, cartilage erosion, and bone destruction from 0 to 3 scores, respectively. Scoring was proportional to the joint damage (Fig. 2B). From the results, it can be seen that WTF, ZCW and MH groups exhibited significant therapeutic effects.

Metabonomic study to assess the holistic efficacy of the WTF

Biomarker characterization in a urinary metabonomic study. Typical urinary base peak intensity chromatograms obtained in positive and negative ion modes are shown in Fig. 3. As an

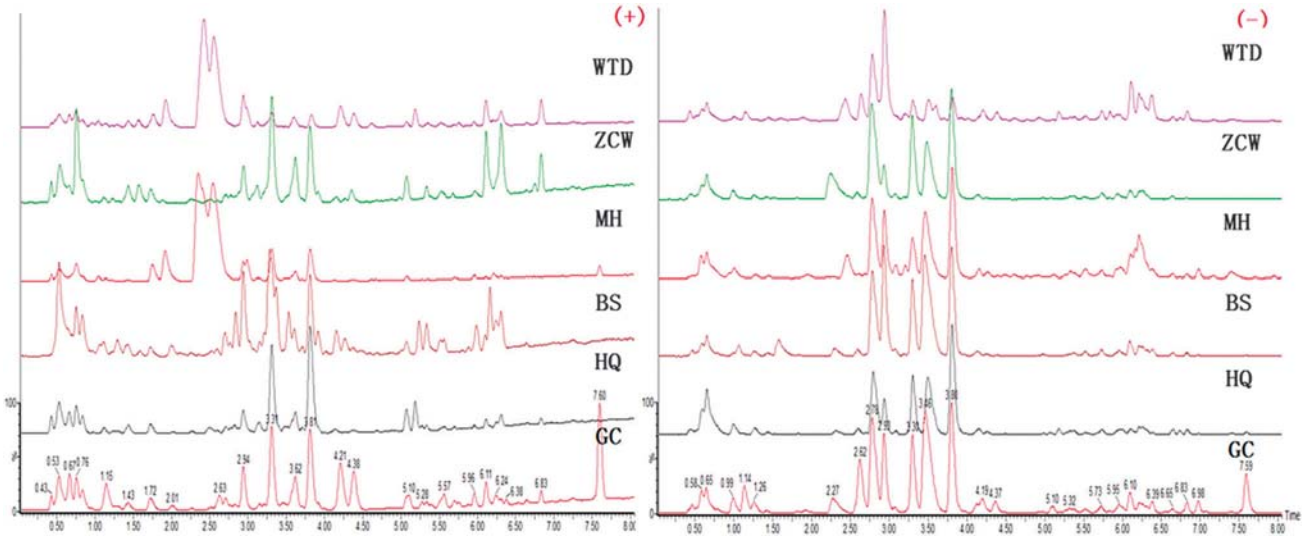

Fig. 3 The typical urinary base peak intensity (BPI) chromatograms. The left BPI chromatograms were the treatment groups in the positive ion mode and the right BPI chromatograms were the treatment groups in the negative ion mode.

unsupervised pattern recognition method, principle component analysis (PCA) was used to construct a model to reduce the data dimensions, and to eliminate coexisting as well as overlap chemical information. With the PCA analysis, different sample groups were classified and the abnormal points were found out. As shown in Fig. 4, different groups have evident variations after administration of each herb for 35 days, and the distance far from the AIA group or the HG group gave an intuitive result which indicated the trends of the therapeutic effect. However, the PCA method has some defects, such as it is vulnerably influenced by the data scale. Thus, orthogonal projection to latent structure squares-discriminant analysis (OPLS-DA) as a supervised method was used to classify the samples correctly, eliminate the unreasonable assumptions and further optimize the model. The intensities of metabolites identified in first 8 min of each TIC were compared for observing the metabolic alteration. The potential biomarkers were identified by comparing the model group and treatment groups, and the variable importance in the projection (VIP) values of these biomarkers was greater than 1.0. The precise molecular masses and fragment ions obtained from UPLC-HDMS of the potential biomarkers and corresponding reference standard compounds were searched in HMDB (http://www.hmdb.ca/spectra/ms/search), of which, these markers were conformed by MassBank (http://www.massbank.jp/) and ChemBank (http://chembank.med.harvard.edu/). Following these thresholds above, 26 endogenous metabolites in urine were recognized to be potential biomarkers, which were related to the influence of the WTF on RA rats (Table 2). Subsequently, the

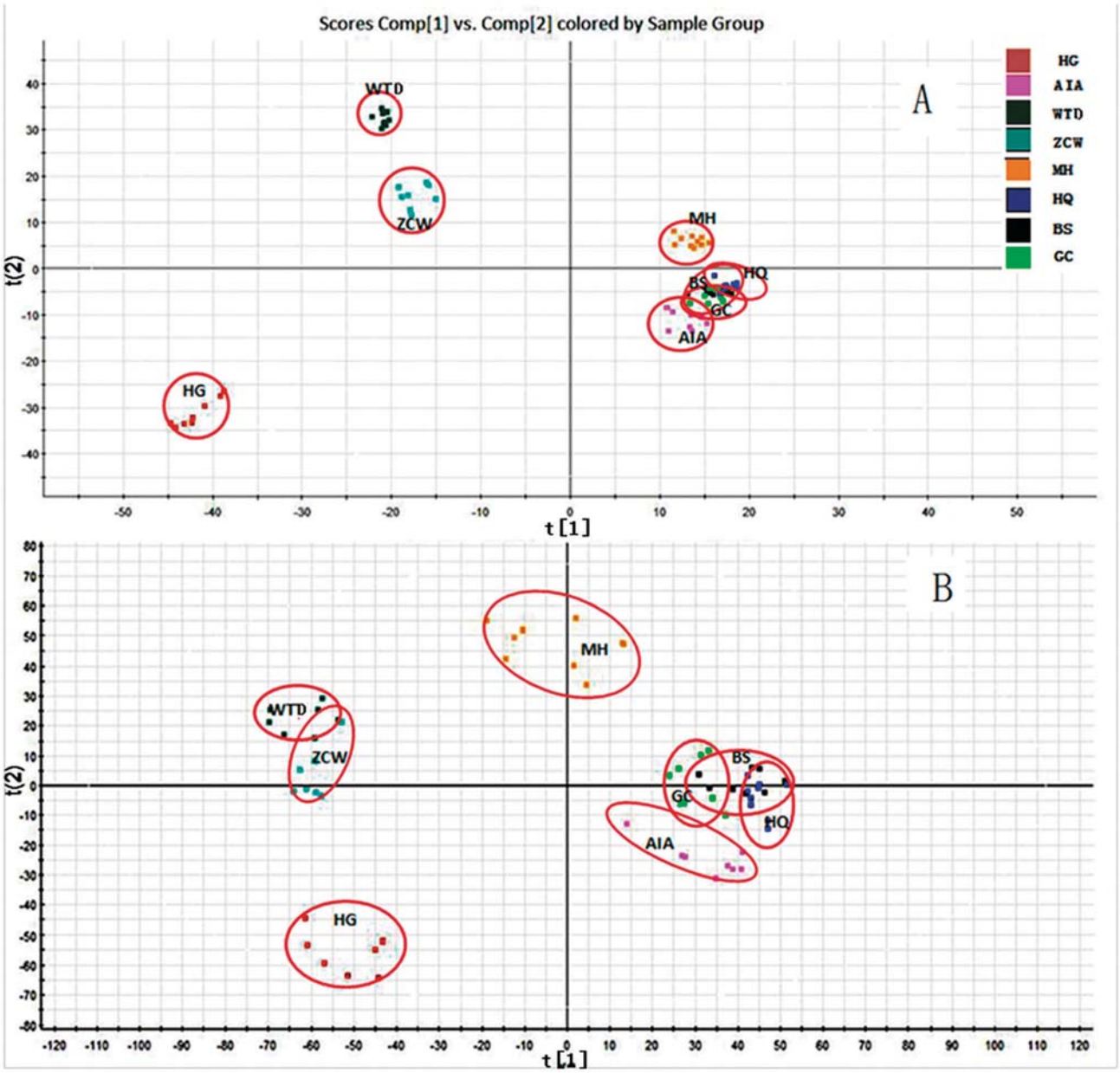

Fig. 4 PCA score plots of rat urine metabolic profiles of WTF, ZCW, MH, HQ, BS, GC, HG and AIA groups. (A) positive ion mode; (B) negative ion mode.

Table 2 Potential biomarkers identified in WTF treatment group compared to model group and found in each monotherapy group

Mode	Rt (min)	VIP value	Accurate mass	Measured mass	Error (ppm)	Formulae	Biomarkers	Found in different group				
ESI+	3.10	1.26	166.0863	166.0866	2.1	$C_9H_{11}NO_2$	L-Phenylalanine	ZCW				
	1.28	1.25	190.0499	190.051	6.0	$C_{10}H_7NO_3$	Kynurenic acid	ZCW	MH	HQ		
	3.80	1.25	250.0475	250.0471	−1.6	$C_8H_{12}NO_6P$	Pyridoxine 5′-phosphate*			HQ		
	3.20	1.23	340.1027	340.104	3.8	$C_{15}H_{17}NO_8$	5-Hydroxy-6-methoxyindole glucuronide				BS	GC
	3.01	1.13	180.0655	180.0663	4.3	$C_9H_9NO_3$	Hippuric acid	ZCW	MH	HQ	BS	
	3.20	1.11	164.0706	164.0714	4.8	$C_9H_9NO_2$	3-Methyldioxyindole					GC
	3.30	5.13	216.0632	216.0637	2.5	$C_5H_{14}NO_6P$	Glycerylphosphorylethanolamine	ZCW		HQ		GC
	3.30	3.34	194.0812	194.0819	3.8	$C_{10}H_{11}NO_3$	2-Methylhippuric acid*	ZCW	MH		BS	GC
	3.30	2.05	76.0393	76.0392	−1.4	$C_2H_5NO_2$	Glycine*		MH			GC
	0.77	2.05	136.0397	136.0397	0.0	$C_4H_9NO_2S$	Homocysteine*	ZCW		HQ	BS	GC
	0.63	1.95	166.0723	166.0732	5.2	$C_6H_7N_5O$	3-Methylguanine	ZCW	MH		BS	GC
	0.61	1.78	114.0662	114.0668	5.4	$C_4H_7N_3O$	Creatine	ZCW	MH			
	0.65	2.80	144.1019	144.1026	4.8	$C_7H_{13}NO_2$	Proline betaine	ZCW		HQ		GC
	2.60	1.85	206.0448	206.046	5.9	$C_{10}H_7NO_4$	Xanthurenic acid	ZCW	MH	HQ		
ESI−	3.80	1.67	175.0248	175.0257	5.1	$C_6H_8O_6$	Ascorbic acid		MH		BS	
	3.97	1.24	173.0819	173.0832	7.3	$C_8H_{14}O_4$	Suberic acid	ZCW	MH	HQ		GC
	5.97	1.97	201.1132	201.1128	−2.2	$C_{10}H_{18}O_4$	Sebacic acid		MH			
	3.49	5.59	107.0502	107.05	−2.2	C_7H_8O	*p*-Cresol					
	0.80	1.30	182.0459	182.0468	5.0	$C_8H_9NO_4$	4-Pyridoxic acid					
	0.51	1.15	124.0074	124.0078	3.3	$C_2H_7NO_3S$	Taurine					
	2.88	2.03	273.00	273.00	1.4	$C_6H_{11}O_{10}P$	D-Glucuronic acid 1-phosphate		MH			
	3.08	1.68	222.0772	222.0784	5.5	$C_{11}H_{13}NO_4$	*N*-Acetyl-L-tyrosine					
	0.70	1.35	191.0197	191.02	1.4	$C_6H_8O_7$	Citric acid	ZCW	MH	HQ		GC
	3.80	8.39	283.0823	283.0837	4.9	$C_{13}H_{16}O_7$	*p*-Cresol glucuronide	ZCW	MH		BS	GC
	2.79	5.54	212.0023	212.0031	3.8	$C_8H_7NO_4S$	Indoxyl sufate		MH	HQ	BS	GC
	3.49	5.59	187.0071	187.0077	3.5	$C_7H_8O_4S$	*p*-Cresol sulfate	ZCW	MH		BS	

Note: biomarkers with asterisk are interfered by WTF.

levels of the above 26 potential biomarkers were analyzed in the five single herb treatment groups to study the metabolic pathways based on their respective roles in the WTF.

Metabolic pathway analysis. MetPA (http://metpa.metabolomics.ca) is a web-based metabonomic tool used to perform pathway analysis and visualization of quantitative metabonomic data. With the potential biomarkers being imported, a holistic pathway analysis can be carried out and the important metabolic pathways can be intuitively displayed. Table 3 shows the main metabolic pathways affected by treating AIA rats using the WTF and each single herb. Also, the impact factor of the WTF acting on AIA rats is listed in this table. Correlation networks of the main potential biomarkers in response to the therapeutic effects of the WTF on AIA rats are intuitively described in Fig. 5. Under the name of the potential biomarkers, one rectangular strip is composed of five different colors respectively to represent the five single herb groups, and the length of each colored bar suggests the relative treatment

Table 3 The main pathway affects by WTF and the five single herbs acted on RA

Pathway name	Group					
	WTF (impact factor)	ZCW	MH	HQ	BS	GC
Phenylalanine, tyrosine and tryptophan biosynthesis	0.50000	▲				
Taurine and hypotaurine metabolism	0.42857					
Phenylalanine metabolism	0.40741	▲				
Valine, leucine and isoleucine biosynthesis	0.33333					
Glyoxylate and dicarboxylate metabolism	0.29630	▲	▲	▲		▲
Glycine, serine and threonine metabolism	0.29197		▲			▲
Pentose and glucuronate interconversions	0.27273				▲	▲
Citrate cycle (TCA cycle)	0.08044		▲	▲		▲
Primary bile acid biosynthesis	0.05952		▲			▲
Vitamin B6 metabolism	0.04902			▲		
Tryptophan metabolism	0.04230					
Starch and sucrose metabolism	0.03778				▲	▲
Glycerophospholipid metabolism	0.02315	▲		▲		▲
Purine metabolism	0.02077					
Arginine and proline metabolism	0.01198	▲	▲			
Glutathione metabolism	0.00573		▲			▲
Alanine, aspartate and glutamate metabolism	0.00316					

Note: the black triangle (▲) marked in a single herb group indicates that the corresponding metabolic pathway has been affected.

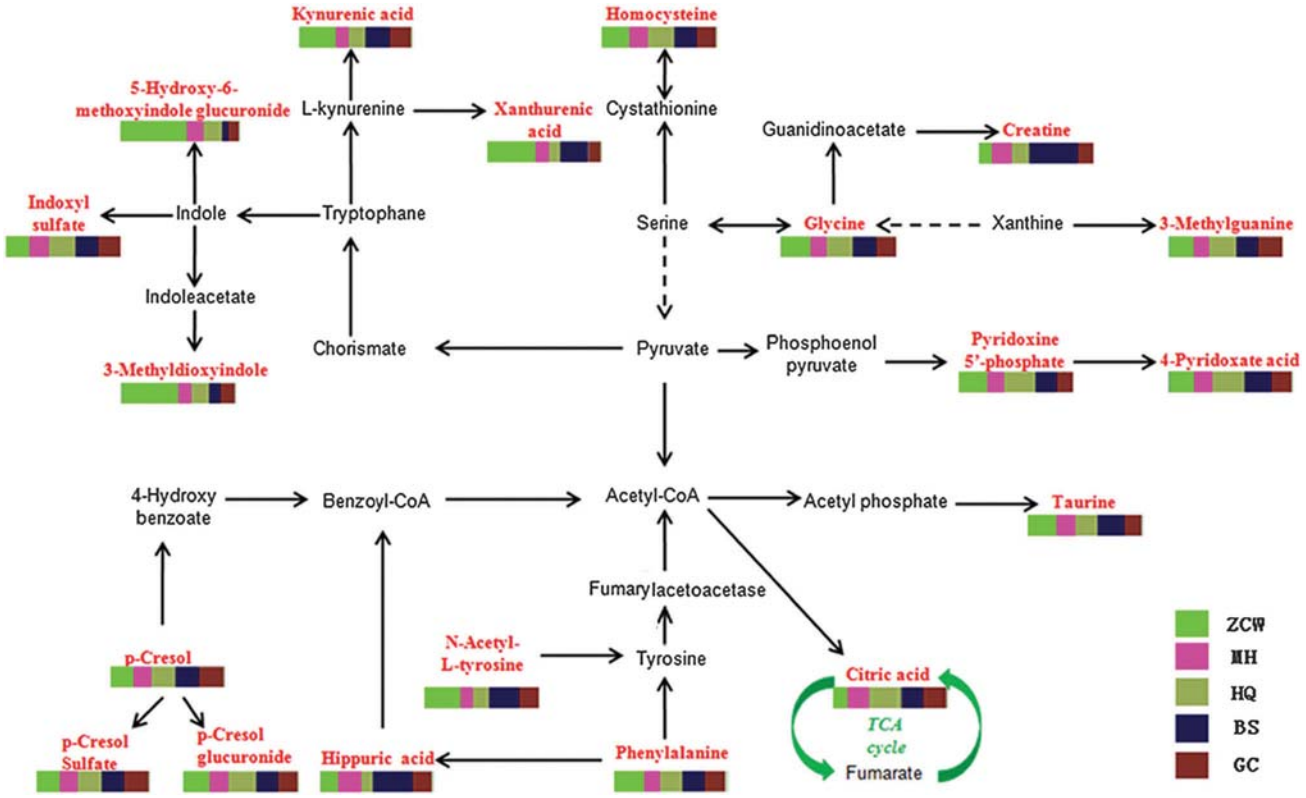

Fig. 5 Correlation networks of main potential biomarkers in response to the therapeutic effects of WTF on AIA rats. The identified potential biomarkers are marked as red; one rectangular strip under the potential biomarker contains five different colors respectively corresponding to the five single herbs, and the length of a color is the relative influence of a herb.

influence of the herb on AIA rats (the length/percentage was obtained by averaging the relative concentrations of the five single treatment groups).

Markers affected by WTT treatment. Obviously, the biomarkers were found by comparison with the WTF treatment group and the AIA model group, and the level of the potential biomarkers in urine could not be always regulated to the normal level. These markers might be disturbed by WTF interference, including pyridoxine 5′-phosphate, 2-methylhippuric acid, glycine and homocysteine (see Table 2).

Markers related to WTF therapeutic effects. All the change trends of the potential biomarkers in different groups are shown in Fig. 6. Vitamin C is considered as an antioxidant and functions as a reducing agent and coenzyme in several metabolic pathways. The ability of vitamin C to donate electrons also makes it a potent water-soluble antioxidant that readily scavenges free radicals, such as molecular oxygen, superoxide, hydroxyl radicals and hypochlorous acid. Lamers *et al.* aimed to identify a metabolic fingerprint for osteoarthritis (OA), and they found that vitamin C could correct metabolic abnormalities after interventional therapy, which means that vitamin C had a noticeable effect on the development of OA.[15] In this study, we noticed that the vitamin C level decreased in the AIA group compared with the HG group, while the content increased after treated by WTF. Among all the single herbs, the most important component is ZCW followed by MH to compare with the other herbs in the formula (see Fig. 6). Phenylalanine, an essential amino acid of humans, plays an important role in the body, whose precursor is tyrosine, or dopamine, or norepinephrine or adrenaline. Phenylalanine is converted to hippuric acid by the metabolism of intestinal bacteria and then excreted as hippuric acid in urine. It could be observed that an increase of phenylalanine concentration along with a decrease of hippuric acid content in the model group indicates that the AIA group had a lower intestinal bacterial activity compared with the normal group. The change levels of these two markers indicated that the phenylalanine pathway had been disturbed in AIA rats. Certain therapeutic effects appeared after treatment with the WTF and single herbs, here the WTF showed a significant effect in regulating the two biomarker contents. In the five single herb treatment groups, the relative influences of ZCW, MH, HQ, BS and GC to regulate phenylalanine were 27%, 14%, 20%, 20% and 19%, and the relative influences to regulate hippuric acid were 16%, 20%, 11%, 36% and 17%, respectively. The results indicated that BS and ZCW mostly contributed to affect intestinal bacteria metabolism in the WTF.

P-Cresol, *p*-cresol glucuronide and *p*-cresol sulfate (PCS) are the major uremic toxins and the latter two compounds mainly circulate the metabolite of *p*-cresol through the intestinal membrane or are glycosylated in the liver. *P*-Cresol plays an important role in the immunodeficiency of uremia.[16] Renal tubular secretion is the main way to clear them from the body.

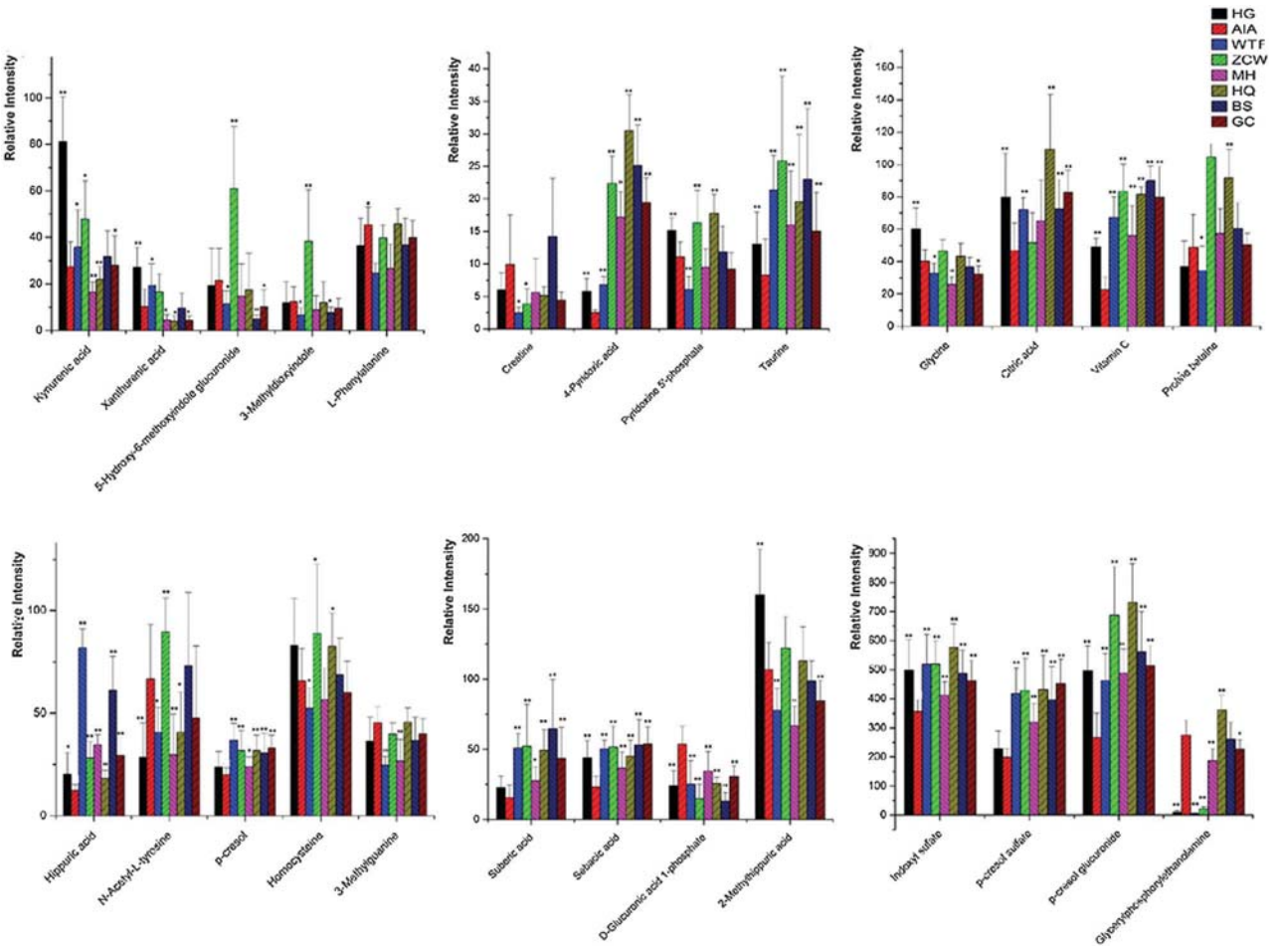

Fig. 6 Relative intensities of potential biomarkers in different groups. (*$p < 0.05$, **$p < 0.01$, compared to the model group).

These compounds could accumulate in the blood with decreasing excretion due to kidney failure.[17,18] As a systemic inflammatory disorder disease, RA is always associated with renal injury and toxin accumulation.[1] In this research, compared with the model group, the level of *p*-cresol and its metabolites in all treatment groups increased obviously. The results showed that the WTF could increase the secretion of these toxic substances, and the five single herbs are effective in promoting the toxins excreted from the body (see Fig. 6).

Tryptophan (TRP) metabolism impacts the immune system mainly *via* the kynurenine pathway, in which indoleamine-2,3-dioxygenase (IDO) plays a pivotal role as a rate-limiting enzyme. Kynurenic acid could reduce *N*-methyl-D-aspartate receptor (NMDAR) activity and disturb brain functions as a neuroprotective molecule.[19] It has been demonstrated that kynurenic acid is attributed to inhibit synovial proliferation in RA.[20] However, both xanthurenic acid and kynurenic acid are downstream products of the kynurenine pathway, down-regulating in model rats and little fine-tuned after different treatment groups. From Fig. 6, it can be seen that ZCW as a main herb exhibited a good effect even better than the WTF. The quantitative analysis results obtained by us indicate that not only the quantities of xanthurenic acid and kynurenic acid, but also the ratio between these two compounds is important, which is the possible mechanism of the WTF for treating RA, which might be due to the decrease of the activity of IDO.[7] The evidence obtained by scientists to date indicates that inflammation symptoms are highly related to the increase of IDO activity.[21,22] 3-Methyl-dioxyindole is a product from TRP metabolism;[23] the amount of that compound increased significantly in urine of the ZCW group, which indicates that the disorder of tryptophan metabolism was mainly caused by ZCW. It can be verified from Fig. 5 that tryptophan metabolism was affected significantly by ZCW, since the longest color in the rectangular color strip under the markers is ZCW.

Glucuronidation usually occurs in the liver, the toxic substances bonded to glucuronic acid have high water solubility and thereby they are more readily excreted from the body. 5-Hydroxy-6-methoxyindole glucuronide is produced from tryptophan metabolism. In this work, the level of 5-hydroxy-6-methoxyindole glucuronide increased in the model group, which might be due to some indole compounds excreted into urine after glucuronidation. From Fig. 6, it can be seen that the WTF could significantly reduce the level of 5-hydroxy-6-methoxyindole glucuronide compared with the model group. Of interest, the five single herbs showed different regulation trends, such as ZCW could increase

the concentration of 5-hydroxy-6-methoxyindole glucuronide significantly in urine, but BS and GC revealed a diametrically opposite function. ZCW though play an important role in WTF, its toxicity still harmful to the body. However, BS and GC through the interactions with other herbs could reduce the toxicity of ZCW and strengthen its effect.

Taurine, synthesised *via* cysteine and vitamin B6, is an essential amino acid and acts as the next most important inhibitory neurotransmitter in the brain. The biological functions of taurine include anti-oxidation, Ca^{2+} transport regulation, and anti-inflammation. And more, taurine chloramine could suppress the secretion of TNF-α *in vivo*.[24] After ranking the impact factor obtained from MetPA, the taurine and hypotaurine metabolism pathway was the next major metabolic pathway in the WTF for treating AIA rats. By the relative content analysis (Fig. 6), all the herbs could improve the low contents of taurine in the model group after treatment, in which ZCW made a greater contribution.

4-Pyridoxic acid and pyridoxine 5′-phosphate are metabolites of vitamin B6, both the compounds decreased in urine of model rats. A report suggested that patients with RA required a higher vitamin B6 supplement than normal healthy population, so as to repair the subnormal vitamin B6 level.[25] Vitamin B6 is closely related to the metabolism of amino acids because it acts as a coenzyme in decarboxylation and transamination. From the pathway analysis results (Table 3), we can see that HQ obviously played a vital role in the vitamin B6 metabolic pathway. HQ was enriched with flavonoid glycosides, triterpene saponins and astragalus polysaccharides which respectively showed significant cellular immune and anti-tumor functions.[26–28] It has been reported that astragaloside IV exhibits antiarthritic activity by suppressing macrophage activation and decreasing IL-1β concentration to alleviate cartilage and bone destruction in AIA rats.[29] Astragalus polysaccharides could strongly suppress NF-κB activation and down-regulate TNF-α and IL-1β expressions in cell experiment.[30] As far as we know, it is the first report on HQ attenuated arthritis by impacting the vitamin B6 metabolic pathway *in vivo*.

Citric acid is formed in the tricarboxylic acid (TCA) cycle, which is associated with energy metabolism.[31] The level of urinary citrate excretion was used as a criterion to diagnose kidney stones, and hypocitraturia was often regarded as renal tubular acidosis and bone disease.[32] The decreased level of citric acid (see Fig. 6) suggested the break down of an aberrant energy metabolism in the model group. After treatment, the WTF could modulate the downward trend of the level in urine. Among the five single herbs, HQ showed a good effect on energy metabolism, probably because of the enhancement of vital Qi corresponding to the related theory on TCM.

Glycerylphosphorylethanolamine (GPE) is a growth stimulant for hepatocytes, which exhibits potential ability to recover astrocytes, and could protect these astrocytes from inflammation, gliosis and neurodegeneration. The bio-function of astrocytes is mainly related to chronic inflammation, and these cells over produce inflammatory cytokines and mediators, exacerbating the progression of many neuropathologies.[33] This is the first report on GPE used as a biomarker in inflammation diseases. The results obtained by us showed that the obvious increase of GPE in model rats might cause the growth of astrocytes, and thus probably increase the secretion of inflammatory cytokines. The WTF showed a significant treatment effect in reducing the content of GPE in urine; ZCW combined with other herbs in this formula played an important role. Suberic acid and sebacic acid are saturated straight-chain dicarboxylic acids with 8 or 10 carbon atoms, respectively, and are present in the urine of patients with fatty acid omega-oxidation disorders.[34] The decreased levels of suberic acid and sebacic acid in the urine of the model group were possibly due to the oxidation disorders of fatty acid. All the treatment groups exhibited excellent regulation effects, since they could up-regulate the content of the two dicarboxylic acids in urine except for the MH treatment group.

Recently, Zhang *et al.* reported a theoretical research study that combined system biology and network pharmacology to elucidate the pharmacological mechanisms of the WTF for treating RA.[35] Based on the results obtained in this research, they concluded that Aconiti Radix Preparata exhibited the most important common effect on Ephedrae Herba, while the less common effect on Glycyrrhiza Radix Preparata and Paeoniae Radix Alba. In this study, the metabolomic method as a powerful approach was used to distinguish the therapeutic effects among different treatment groups (see the PCA score of Fig. 4). Equally, by the comprehensive evaluation of biochemical parameters, histopathologic assessment, biomarker and metabolic pathway analysis, one could conclude that ZCW played a major role in the treatment of RA using the WTF followed by MH. HQ, BS and GC displayed synergistic actions to facilitate the effectiveness. To our knowledge, this is the most comprehensive research to elucidate the therapeutic effects of different herbs in the WTF for treating RA.

Conclusion

Nowadays, TCM shows significant advantages in the treatment of chronic diseases. As an important part of TCM, the formula possessing multi-target and multi-component characteristics always presents good curative effects. In this paper, a pharmacodynamics and urinary metabonomic study was performed to assess the efficacy of the WTF and the single herbs on AIA model rats. The results revealed that different herbs in the WTF disturbed different metabolic pathways, and the main metabolism pathways of the WTF for treating RA were the phenylalanine, tyrosine and tryptophan biosynthesis pathway; the taurine and hypotaurine metabolism pathway; and the phenylalanine metabolism pathway. ZCW as the important component mainly affected the phenylalanine, tyrosine and tryptophan biosynthesis pathway; the phenylalanine metabolism pathway; and the glyoxylate and dicarboxylate metabolism pathway. MH as the ministerial component mainly affected the glyoxylate and dicarboxylate metabolism pathway; the glycine, serine and threonine metabolism pathway; and the citrate cycle metabolism pathway. ZCW and MH showed good treatment effects on

RA, while HQ, BS and GC displayed synergistic actions to facilitate the effectiveness.

Materials and methods

Materials

Aconiti Radix Preparata, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata were purchased from Ji Lin Pharmacy. All the herbs met the standards recorded in Chinese Pharmacopoeia (2010 Edition) and identified by Prof. Shumin Wang (Changchun University of Chinese Medicine). Complete Freund's adjuvant (CFA) was taken from Chondrex, Inc. (Redmond, WA, USA). L-Phenylalanine, kynurenic acid, glycine, homocysteine, xanthurenic acid, 2-methylhippuric acid, ascorbic acid, suberic acid, sebacic acid, taurine, *N*-acetyl-L-tyrosine, citric acid, hippuric acid were supported by Sigma-Aldrich (St. Louis, Mo, USA). Leucine enkephalin and sodium formate were obtained from Waters (Milford, USA). Acetonitrile and formic acid, HPLC-grade, were obtained from Fisher Scientific (Loughborough, UK). Ultrapure water was prepared using a Milli-Q plus (Milford, MA, USA). Rat IL-1β ELLSA kits, TNF-α ELLSA kits, superoxide dismutase (SOD) kits and malondialdehyde (MDA) kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Extractions

The preparation method of WTF extraction was described in a previous report:[9] a total of 900 g crude herbs, including Aconiti Radix Preparata, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata at the 2 : 3 : 3 : 3 : 3 ratios were immersed in 9 L of water for 1 h, and then heated to reflux for 1.5 h. The extraction solution was filtered and the drugs were put into another 7.2 L of water and refluxed for 1.5 h. All the filtered extraction solutions were combined and concentrated to 600 mL. Aconiti Radix Preparata, Ephedrae Herba, Paeoniae Radix Alba, Astragali Radix and Glycyrrhiza Radix Preparata were extracted using the procedure of WTF extraction, and concentrated to 1.5 g mL^{-1}, respectively.

AIA model and treatment

Male Sprague-Dawley rats (weights 200 ± 20 g) were supplied by Dalian Medical University (Dalian, China). All rats were randomly divided into 8 groups respectively containing 10 rates, namely the healthy control group (HG), the model control group (AIA), the WTF treatment group (WTF), the Aconiti Radix Preparata treatment group (ZCW), the Ephedrae Herba treatment group (MH), the Paeoniae Radix Alba treatment group (BS), the Astragali Radix treatment group (HQ) and the Glycyrrhiza Radix Preparata treatment group (GC). Rats were housed under standard laboratory conditions (temperature, 22 ± 2 °C; relative humidity, 50 ± 5%), and food and water were supported *ad libitum* throughout the whole experiment. The rats were accommodated for 1 week and then fed in metabolism cages for another 24 h before initiating the experiment. All rats were fasted 24 h before collecting urine and sacrificing. The rats in the model and treatment groups were respectively injected 0.1 mL of CFA in the left hind digits while the rats in the control group were injected 0.1 mL 0.9% saline solution at the same time. The AIA model was constructed after 2 week immunization. The rats in the WTF treatment group were administered intragastrically with WTF extraction at a dose of 9.8 g crude drug per kilogram per day (equal to 10 mL per kg per day) as reported in a previous study;[9] the ZCW treatment group was administered intragastrically with Aconiti Radix Preparata extraction at a dose of 1.4 g crude drug per kilogram per day (equal to 10 mL per kg per day); MH, BS, HQ and GC treatment groups were administered intragastrically with each extraction at a dose of 2.1 g crude drug per kilogram per day (equal to 10 mL per kg per day). The rats in the healthy control and AIA model group were administered with distilled water 2 mL per day. All the rats were administered for 3 weeks. All the experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Sample collection and preparation

The serum sample was obtained from the whole blood after 21 days of administration, upon centrifugation at 3500 rpm at 4 °C for 10 min to get the supernatant. The serum samples were stored immediately at 80 °C. Prior to biochemical parameter analysis, the serum samples were thawed at 4 °C. The levels of TNF-α and IL-1β in serum were determined using commercial ELISA kits. SOD and MDA levels were determined by using commercial SOD and MDA kits.

The right hind joints were kept after the rats were sacrificed, and soaked in 10% formalin solution for a week to fix the joint, then decalcified in Gooding and Stewart's solution for two weeks. Joint sections were stained with haematoxylin and eosin (H&E) for general evaluation. A total of 16 grades of the evaluation were established, for inflammatory infiltration, synovial proliferation, cartilage erosion, and bone destruction graded from 0 to 3 for each aspect as previously reported.[36]

Samples of 24 h urine of rats were collected weekly from the metabolism cages, and a test tube with an ice pack was used so as to keep the urine at a low temperature. The urine was centrifuged at 12 000 rpm at 4 °C for 10 min to remove particle contaminants, and the supernatant was stored at −80 °C. The supernatant was thawed at 4 °C and diluted with 10 times of ultrapure water, and then filtered through a 0.22 μm filter membrane before UPLC-MS analysis.

UPLC-MS conditions

Chromatographic analysis was performed using a Waters Acquity UPLC system coupled with a Q-TOF SYNAPT G2 High Definition Mass Spectrometer (Waters, UK). The separation was carried out by a Waters ACQUITY UPLC BEH C18 Column (2.1 mm × 50 mm, 1.7 μm) at 40 °C. An aliquot of 5 μL of sample solution was injected into the column for each run. The mobile phase consisted of a linear gradient of A (0.1% formic acid in water) and B (acetonitrile) with a constant flow rate of 0.3 mL min^{-1}. The gradient program was optimized as follows: 5% B at 0–1.6 min, 5–15% B at 1.6–2 min, 15% B at 2–3.8 min, 15–35% B at 3.5–5.5 min, 35–40% B at 5.5–6 min, 40–100% B at

6–8 min. The sample room was maintained at 4 °C during the whole analysis. By comparing with the peak intensity, optimal conditions of the mass spectrometer were set (source temperature at 120 °C, desolvation gas temperature at 350 °C, cone desolvation gas flow rate at 50 L h^{-1}, desolvation gas flow rate at 700 L h^{-1}). For the positive ion mode, the capillary voltage was 3.0 kV, the cone voltage was 30 V and the extraction cone voltage was 5.0 V. For the negative ion mode, the capillary voltage was 2.5 kV, the cone voltage was 30 V and the extraction cone voltage was 5.0 V. MS data were centroided in the full-scan mode from 50 to 1000 Da with a 0.3 s scan time and a 0.1 s inter scan delay. Sodium formate was used to set up mass spectrometer calibration. Leucine enkephalin (2 ng mL^{-1}) was used as the lockspray at a flow rate of 5 μL min^{-1}, the accurate molecular weight was 554.2615 and 556.2771 in the negative and the positive mode, respectively. Argon was used as the collision gas. The MS^E mode was applied to obtain the fragment information of potential biomarkers. The energy was first kept at 25 eV, and was regulated depending on the fragmentation.

Urine sample analysis

As is necessary to optimize the UPLC-Q-TOF-HDMS conditions and access the data quality before and during the analysis, the "quality control" (QC) sample was needed. Therefore all the urine samples, 100 μL of each, were mixed as the QC sample. The run order of the entire sample set was random. Before analysis, 5 QC samples were run to equilibrate the system, and then a QC sample was injected at a regular interval (every ten samples) throughout the analytical run to observe the repeatability and stability of the system.[37]

Pattern recognition analysis and data processing

The UPLC Q-TOF MS raw data were first processed by MarkerLynx Application Manager and MassLynxV4.1 for compound detection and peak alignment. After the first processing, the data complexity was reduced and the interferences were eliminated. Then the exact mass, retention time and the intensities as a data matrix were imported into EZinfo 2.0, a multivariate statistical analysis software. Principle component analysis (PCA) and orthogonal projection to latent structures squares-discriminant analysis (OPLS-DA) were conducted to assess the trend of disease, therapeutic effect and to find the biomarkers. The biochemical data were introduced into PASW Statistics 18.0 software for statistical analysis, and the p value of less than 0.01 was selected as significant statistical differences. Available biochemical databases, such as ChemBank (http://chembank.med.harvard.edu/), MassBank (http://www.massbank.jp/), HMDB (http://www.hmdb.ca/spectra/ms/search), METLIN (http://metlin.scripps.edu/) and KEGG (http://www.kegg.com/) were used to identify potential biomarkers and construct the metabolic pathway.

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References

1 D. M. Lee and M. E. Weinblatt, *Lancet*, 2001, **358**, 903–911.
2 P. E. Lazzerini, P. L. Capecchi, M. Acampa, M. Galeazzi and F. Laghi-Pasini, *Autoimmun. Rev.*, 2014, **13**, 936–944.
3 I. A. M. van den Oever, N. Sattar and M. T. Nurmohamed, *Ann. Rheum. Dis.*, 2014, **73**, 954–957.
4 E. Choy, K. Ganeshalingam, A. G. Semb, Z. Szekanecz and M. Nurmohamed, *Rheumatology*, 2014, **53**, 2143–2154.
5 C. M. Mariaselvam, M. Aoki, S. Salah, W. Boukouaci, H. Moins-Teisserenc, D. Charron, R. Krishnamoorthy, R. Tamouza and V. S. Negi, *Immunobiology*, 2014, **219**, 772–777.
6 K. Bauerova, A. Acquaviva, S. Ponist, C. Gardi, D. Vecchio, F. Drafi, B. Arezzini, L. Bezakova, V. Kuncirova, D. Mihalova and R. Nosal, *Autoimmunity*, 2015, **48**, 46–56.
7 Y. Qi, Z. F. Pi, S. Liu, F. R. Song, N. Lin and Z. Q. Liu, *Mol. BioSyst.*, 2014, **10**, 2617–2665.
8 C. Wang, C. F. Liu, H. Y. Wan, D. H. Wang, D. N. Sun, T. F. Xu, Y. Yang, Y. K. Qu, Y. Xu, X. H. Jing, J. L. Liu, S. P. Chen, Z. Q. Liu and N. Lin, *BioMed Res. Int.*, 2015, DOI: 10.1155/2015/328707.
9 Y. Qi, S. Z. Li, Z. F. Pi, F. R. Song, N. Lin, S. Li and Z. Q. Liu, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2014, **953**, 11–19.
10 Z. J. Zheng, T. M. Yan, W. Y. Chen, L. Ye, L. Tang and Z. Q. Liu, *Xenobiotica*, 2012, **42**, 775–783.
11 J. K. Nicholson, J. C. Lindon and E. Holmes, *Xenobiotica*, 1999, **29**, 1181–1189.
12 C. X. Hu and G. W. Xu, *TrAC, Trends Anal. Chem.*, 2014, **61**, 207–214.
13 D. G. Robertson, M. D. Reily and J. D. Baker, *J. Proteome Res.*, 2007, **6**, 526–539.
14 W. P. Arend and J. M. Dayer, *Arthritis Rheum.*, 1995, **38**, 151–160.
15 R.-J. A. Lamers, J. DeGroot, E. J. Spies-Faber, R. H. Jellema, V. B. Kraus, N. Verzijl, J. M. TeKoppele, G. K. Spijksma, J. T. Vogels and J. van der Greef, *J. Nutr.*, 2003, **133**, 1776–1780.
16 R. Vanholder, R. Desmet, M. A. Waterloos, N. Vanlandschoot, P. Vogeleere, E. Hoste and S. Ringoir, *Kidney Int.*, 1995, **47**, 510–517.
17 Y. Miyamoto, H. Watanabe, T. Noguchi, S. Kotani, M. Nakajima, D. Kadowaki, M. Otagiri and T. Maruyama, *Nephrol., Dial., Transplant.*, 2011, **26**, 2498.
18 B. Bammens, P. Evenepoel, H. Keuleers, K. Verbeke and Y. Vanrenterghem, *Kidney Int.*, 2006, **69**, 1081.
19 L. R. Kolodziej, E. M. Paleolog and R. O. Williams, *Amino Acids*, 2011, **41**, 1173–1183.
20 J. Parada-Turska, W. Rzeski, W. Zgrajka, M. Majdan, M. Kandefer-Szerszen and W. Turski, *Rheumatol. Int.*, 2006, **26**, 422–426.
21 M. Maes, R. Verkerk, S. Bonaccorso, W. Ombelet, E. Bosmans and S. Scharpe, *Life Sci.*, 2002, **71**, 1837–1848.

22 X. Zheng, A. Kang, C. Dai, Y. Liang, T. Xie, L. Xie, Y. Peng, G. Wang and H. Hao, *Anal. Chem.*, 2012, **84**, 44–51.
23 H. Dong, A. Zhang, H. Sun, H. Wang, X. Lu, M. Wang, B. Ni and X. Wang, *Mol. BioSyst.*, 2012, **8**, 1206–1221.
24 M. Imae, T. Asano and S. Murakami, *Amino Acids*, 2014, **46**, 81–88.
25 E. P. I. Chiang, J. Selhub, P. J. Bagley, G. Dallal and R. Roubenoff, *Arthritis Res. Ther.*, 2005, **7**, R1404–R1411.
26 Y. Qi, S. Z. Li, Z. F. Pi, F. R. Song, N. Lin, S. Liu and Z. Q. Liu, *Talanta*, 2014, **118**, 21–29.
27 P. Zhao, G. H. Su, X. Y. Xiao, E. K. Hao, X. L. Zhu and J. Ren, *Toxicol. Lett.*, 2008, **182**, 29–35.
28 S. Li, F. L. Bian, L. Yue, H. Jin, Z. G. Hong and G. W. Shu, *Int. J. Biol. Macromol.*, 2014, **69**, 64–72.
29 B. Wang and M. Z. Chen, *Arch. Pharmacal Res.*, 2014, **37**, 793–802.
30 X. J. He, J. Shu, L. Xu, C. Lu and A. P. Lu, *Molecules*, 2012, **17**, 3155–3164.
31 M. Akram, *Cell Biochem. Biophys.*, 2014, **68**, 475–478.
32 C. Renata, V. Fabio, B. Angela and S. Sergio, *Front. Biosci.*, 2003, **8**, S1084–S1106.
33 M. Bisaglia, V. Venezia, M. Biglieri, C. Russo, F. Mancini, C. Milanese and G. Schettini, *Neurochem. Int.*, 2004, **44**, 161–170.
34 N. Gregersen, P. B. Mortensen and S. Kolvraa, *Pediatr. Res.*, 1983, **17**, 828–834.
35 Y. Zhang, D. Wang, S. Tan, H. Xu, C. Liu and N. Lin, *J. Evidence-Based Complementary Altern. Med.*, 2013, 548498.
36 N. L. Chillingworth and L. F. Donaldson, *J. Neurosci. Methods*, 2003, **128**, 45–52.
37 E. J. Want, E. Holmes, I. D. Wilson, H. Gika and J. K. Nicholson, *Nat. Protoc.*, 2010, **5**, 1005–1018.